·CLINICAL RESEARCH·

Expression of Epithelial Cadherin in Early Gastric Cancer and Its Correlation to Lymph Node Micrometastasis and Clinicopathologic Features

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[ABSTRACT] BACKGROUND & OBJECTIVE: Lymph node micrometastasis in early gastric cancer is being widely discussed. Cytokeratin (CK) staining is an important way to distinguish epithelial cancer cells. This study was to investigate the correlations of epithelial cadherin (E-cad) expression to lymph node micrometastasis, and clinicopathologic features of early gastric cancer, and to evaluate its clinical significance. METHODS: Morphology of 4522 lymph nodes from 162 patients with early gastric cancer was observed with HE staining and CK immunostaining. E-cad expression in 135 primary lesions of these patients was detected by immunohistochemistry. The correlations of E-cad expression to clinicopathologic features were analyzed. RUSULTS: The detection rate of lymph node metastasis by CK staining was significantly higher than that by HE staining (26.5% vs. 6.8%, $P<0.001$). CK immunostaining detected 32 cases of lymph node micrometastasis which were missed by HE staining. Lymph node micrometastasis was frequently found in primary tumors with a diameter of more than 1.0 cm, in those that were poorly differentiated, deeply invaded (for example, to the submucosa), showed lymphatic or vascular invasion, and in those that showed loss of E-cad expression ($P<0.05$). The reduced expression rate of E-cad in primary tumor was 57.0%, closely correlated to lymph node micrometastasis. The 5-year survival rate was significantly lower in the patients with lymph node micrometastasis than in those without such metastasis (93.6% vs. 100%, $P<0.01$). CONCLUSION: Primary tumor more than 1.0 cm in diameter, poor differentiation, deep invasion, lymphatic or vascular invasion, and loss of E-cad expression are risk factors for lymph node metastasis in early gastric cancer.

KEYWORDS: Gastric neoplasm; Lymph node metastasis; Immunohistochemistry; Epithelial cadherin; Cytokeratin

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1. Introduction

Early gastric cancer refers to the tumor that is confined to the mucosa or submucosa, regardless of the presence or absence of lymph node metastasis [1]. With the wide use of gastrointestinal endoscopy and improved technologies, the diagnosis of gastric cancer becomes increasingly earlier, and postoperative survival rate is significantly improved [2-4]. Although the implementation of the D2 lymphadenectomy for gastric cancer has become the standard procedure for the treatment of early gastric cancer [5,6], some less-invasive procedures without lymphadenectomy have been used for the treatment of some patients with early gastric cancer. Compared to the D2 lymphadenectomy, those less-invasive surgeries not only achieve the same satisfactory therapeutic effect, but also obtain a better postoperative quality of life [7].

Lymph node metastasis is the most important prognostic factor for patients with early gastric cancer. The lymph node micrometastasis of early gastric cancer can be found by CK immunohistochemical staining [10,11]. Epithelial cadherin (E-cad) has been considered to play an important role in the metastasis of epithelial-derived tumors [12], but the clinical significance and the correlation of the expression of E-cad in the primary lesion of early gastric cancer to lymph node micrometastasis remain unclear. In addition, preoperative evaluation of the lymph node involvement in early gastric cancer is always very difficult. Understanding of the clinicopathological features of lymph node micrometastasis is important in choosing a reasonable method of operation. This study was to investigate and assess the correlation of the expression of E-cad in the primary lesion of patients with early gastric cancer to lymph node micrometastasis.

2. Data and Methods

2.1 General information

A total of 162 patients with early gastric cancer who received gastrectomy from Jan. 1986 to Dec. 1990 in Tottori University Hospital were enrolled. There were 98 males and 64 females, aged from 37 to 82 years, with a median age of 63 years. None of the patients underwent preoperative chemotherapy or radiotherapy. Complete gastrectomy was performed in 19 cases (11.7%), distal gastrectomy in 126 cases (77.8%), proximal subtotal gastrectomy in 15 cases (9.3%), and partial resection of the stomach in 2 patients (1.2%). D1 lymphadenectomy was performed in 2 cases, D2 lymphadenectomy in 115 cases, and selective D3 lymphadenectomy in 45 cases. All patients were followed up for 5 years.

2.2 Clinicopathological data

Clinicopathological data were collected from the medical records and evaluated according to Japanese Classification of Gastric Cancer (the 13th edition, June 1999) [1]. Early gastric cancer refers to the primary lesions of gastric cancer confined to the mucosa or submucosa. The tumor size was the longest diameter of the base of the primary lesion. The tumor size was less than or equal to 1 cm in 18 cases, 1-2 cm in 41 cases, and larger than 2 cm in 103 cases. Based on macroscopic observation, there were 23 cases of type 0-I, 27 cases of type 0-Ⅱ, 112 cases of 0-Ⅲ; 107 cases were differentiated (51 cases were well differentiated and 56 cases were moderately differentiated), and 55 cases were poorly differentiated or undifferentiated. HE staining of the tumor invasion was divided into three sub-groups: 84 patients were mucosal cancer (tumor was confined to the mucosa, m-cancer); 49 cases were submucosal cancer 1 (tumor invasion was limited to the upper two-thirds of the submucosa, sm1); 29 cases were submucosal cancer 2 (tumor invasion was more than two-thirds of the submucosa, but did not reach the muscle layer, sm2); 15 patients had lymphatic invasion; and 14 cases had vascular invasion.

2.3 Immunohistochemistry

2.3.1 CK immunohistochemical staining of lymph nodes

A total of 4,522 lymph nodes from 162 patients were collected, with an average of 28 nodes per case (10-61 nodes). The 4 μm-thick serial paraffin sections of the specimens were routinely dewaxed. Hematoxylin & eosin (HE) and/or CK immunohistochemical staining were performed. Forty gastric lymph nodes obtained from 20 benign gastric ulcer patients were used as normal controls; 135 primary lesions of early gastric cancer were used as positive controls. The standard SAB immunohistochemical staining was used for CK staining [13]. After dewaxation and dehydration, 3% hydrogen peroxide methanol solution was added to block endogenous peroxidase activity. Then the sections were incubated with mouse anti-human monoclonal antibody CAM5.2 (Becton Dickinson. San Jose, CA, USA) at 4 ° C overnight, followed by the incubation with rabbit anti-mouse immunoglobulin biotin antibody. Then horseradish enzyme labeled HRP-streptoavidin was applied, and DAB was used for color development. Methylene was used as background staining and PBS as a negative control.

2.3.2 E-cad immunohistochemical staining of the primary tumors

Of the 162 cases with early gastric cancer, 4 μm-think serial paraffin sections of 135 primary tumor tissues were prepared. Not

enough samples were obtained from the rest 27 cases. The expression of E-cad of each case was detected by standard ABC immunohistochemical staining [14]. After dewaxation and dehydration, 3% hydrogen peroxide methanol solution was added to block endogenous peroxidase activity., Sections were incubated with mouse anti-human E-cad monoclonal antibody (Clone HECD-1, 1:100, Takara, Japan) at 4 ° C overnight, and then with rabbit anti-mouse immunoglobulin biotin antibody for 45 minutes, followed by the incubation with ABC solution for 30 min (Vectastain ABC Kit, Vector laboratory, Burlinggame, CA, USA). The sections were then stained using two-benzidine and HE. PBS was used as the negative control. Non-cancerous gastric cells of every primary lesion were used as the internal positive control.

2.4 The evaluations of immunohistochemical staining

At first, pathological specialists made diagnosis based on H&E stained sections, and evaluated the invasive depths and lymph node metastasis. Other experienced doctors evaluated immunohistochemical staining sections. Lymph node micrometastasis was detected by CK staining only, but not by HE staining. The percentage of E-cad staining in cancerous cells was scored from grade 0 to 3 in a semi-quantitative pattern: 0 denoting negative staining (the percentage of stained cells were less than 10%), 1 denoting cytoplasmic staining, 2 denoting heterogeneous staining (the percentage of stained cells were more than 10% , but less than 90%), 3 denoting normal staining (the number of stained cells were more than 90%) [15]. Grade 0, 1 and 2 were regarded as low (abnormal) expression and grade 3 was high (normal) expression of E-cad.

2.5 Statistical analysis

Chi-square test was used to compare the clinicopathologic data with experimental data; survival curves were constructed according to the Kaplan-Meier method; and Wilcoxon signed's log-rank test was used for survival curve analysis; Cox's regression model was used for multivariate analysis; $P<0.05$ was considered as statistically significant.

3. Results

3.1 Lymph node metastasis of early gastric cancer

Of 162 cases, lymph node metastasis was found in 11 cases (6.8%, 11/162) by H&E staining: 1 was out of 84 cases (1.2%, 1/84) of mucosal cancer with lymph node metastasis and 10 were out of 78 cases (12.8%, 10/78) of submucosal cancer with metastasis. Lymph node metastasis was found in 43 cases (26.5%, 43/162. P <0.0001) using CK immunohistochemical staining. Lymph node metastasis rate of intromucosal cancer was 19.0% (15/78), and that of submucosal cancer was 34.6% (27/78). Of the total 4 522 nodes, lymph node involvement rate was 0.5% found by HE staining (22/4 522), and that was 2.5% (112/4 522, P <0.0001) found by CK immunohistochemical staining. Thirty-two cases (19.8% 32/162) of early gastric cancer were found lymph node micrometastasis by CK immunohistochemical staining, but not by HE staining. Of the 16 cases that were found lymph node micrometastases in the mucosal cancer, 12 cases (75.0%) were only found a single cell or scattered cancer cells in the lymph nodes. Of the 27 cases that were found lymph node micrometastases in the submucosal cancer, 19 (70.4%) were found scattered or clustered cancer cells in the lymph nodes (Figure 1A, B). Of all 43 cases with lymph node metastasis, 19 cases (44.2%) had extra-perigastric lymph node metastasis, including 16 cases having group 2 lymph node metastases and 3 cases having group 3 lymph node metastases. In the 40 lymph nodes from patients with benign gastric ulcers, no positive CK staining cells were found.

3.2 The relationship between lymph node metastases and the tumor size of primary lesions

We analyzed the clinicopathologic features of the 43 cases that were found lymph node micrometastases by CK immunohistochemical staining. Among them, micrometastases were found more frequently in the original lesions whose diameters were more than 2.0 cm than those were less than 1.0 cm ($P<0.05$). Of the 27 cases of submucosal cancer that occurred micrometastases, the original lesions of 23 cases (85.2%) were greater than 2.0 cm, and the other 4 cases were less than or equal to 2.0 cm. In the 4 cases, 3 cases had group 2 lymph node micrometastases. Of the 16 cases of mucosal cancer with lymph node micrometastases, the original lesions of 6 cases (38.0%) were less than or equal to 2.0 cm, 5

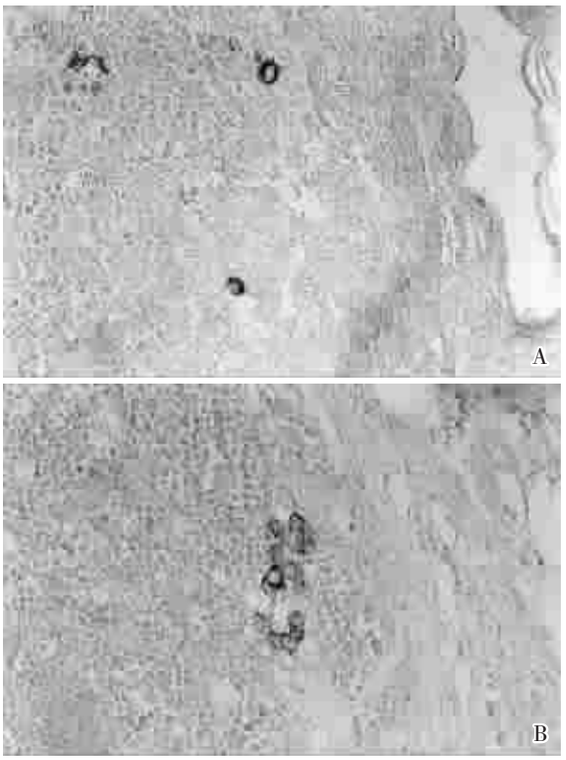

Figure 1 Lymph node micrometastases in early stage gastric cancer (CK ×200)

A: Scattered cancer cells in the lymph node.

B: Clustered cancer cells in the lymph node.

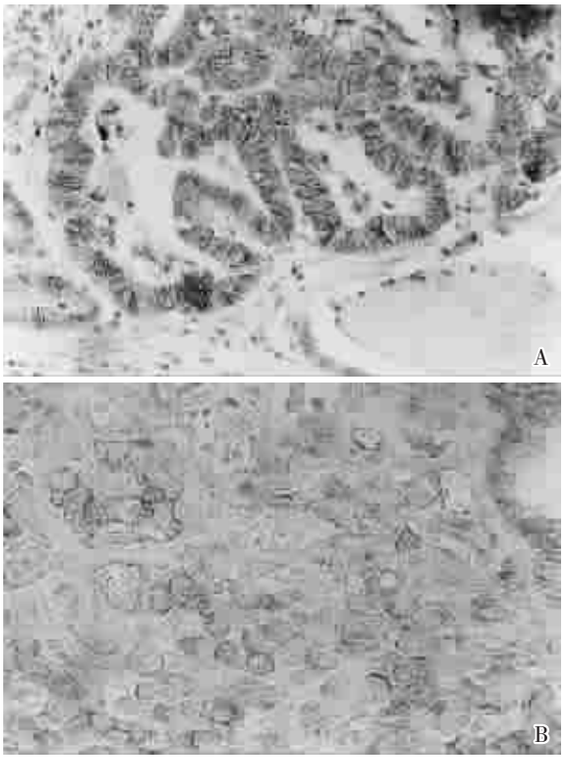

Figure 2 Expression of E-cadherin in cell-cell boundaries of early gastric cancer cells (ABC ×200)

A. Preserved expression of E-cadherin in cancerous mucosa of a primary gastric cancer lesion.

B. Loss of expression of E-cadherin in cancerous mucosa of another primary gastric cancer lesion.

of which had the lesion between 1.0-2.0 cm. Of all the 43 cases, metastases in the form of scattered or clustered cancer cells were found more frequently in tumors larger than 2.0 cm. Tumor differentiation, the depth of the invasion, lymphatic and vascular invasion were strongly associated with lymph node metastasis (P<0.05). Of the 26 cases with discrete or scattered cancer cells in the lymph nodes, the primary tumors of 16 cases (61.5%) were poorly differentiated or undifferentiated (P<0.05). Of all 20 poorly differentiated or undifferentiated tumors, 12 (60.0%) occurred group 2 and/or group 3 lymph node micrometastases (P<0.05).

3.3 E-cad expression in early gastric cancer

Of all 135 cases, normal expression of E-cad was detected at the cell-cell boundaries between the normal mucosa and the adjacent tumors. Low expression of E-cad was found in 77 cases (57.0%) of early gastric cancer patients (Figure 2A, B), and normal expression was present in 58 cases. Negative expression of E-cad was found more frequently in poorly differentiated or undifferentiated tumors (P<0.0001), in the tumor more than 1.0 cm (P<0.05), in deeply invaded tumors (P<0.005), and tumors with lymphatic and vascular invasion (P<0.05). Of 37 cases with CK-positive cells in the lymph nodes, 30 cases (81.1%) showed negative expression of E-cad at the primary sites (P<0.0005). No matter lymph node micrometastases were in the form of a single cell, clustered or scattered cancer cells, the primary lesions usually showed negative expression of E-cad. In those cases with negative E-cad expression, more micrometastatic lymph nodes belonging to group 2 were found (Table 1).

3.4 Prognosis of patients with early gastric cancer

The 5-year survival rate of all 162 patients was 97.5%. Four cased died of recurrence (1 liver metastases and 3 peritoneal metastasis): 2 of them were diagnosed lymph node metastasis by H&E staining, while the other 2 were not diagnosed by H&E staining, but by CK immunohistochemical staining. The 5-year survival of the 119 cases who were not found

Table 2 Logistic regression multivariate analysis of risk factors associated with lymph node metastasis detected by CK immunostaining

Variable	Regression coefficient	*P*
Macroscopic type (elevated, depressed)	0.061	0.489
Ulceration (negative, positive)	0.123	0.284
Histological type (differentiated, undifferentiated)	0.107	0.214
Depth of invasion (mucosal, submucosal)	0.100	0.224
Lymphatic invasion (negative, positive)	0.394	0.010
Vascular invasion (negative, positive)	0.041	0.775
Expression of E-cad (preserved, loss)	0.222	0.006

lymph node metastasis by CK and H&E staining was 100%; while that of the 11 cases who were found lymph node metastases by HE staining was 81.8%; and that of the remaining 32 cases who were found lymph node micrometastases by CK immunohistochemical staining, but not by HE staining was 93.6% (P<0.01, Figure 3).

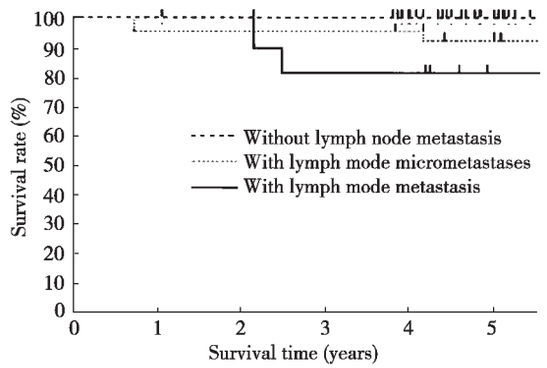

Figure 3 Survival curves of the 162 patients with early gastric cancer

Lymphatic invasion and abnormal expression of E-cad were independent risk factors for lymph node metastases detected by CK immunohistochemical staining (P<0.05, Table 2). The macroscopic type and ulceration of the tumor, but not the lymphatic involvement, were independent prognostic factors (P<0.05).

4. Discussions

It has been a consensus that large tumor, lymphatic invasion and the depth of submucosal invasion are the risk factors of lymph node metastases [2, 3]. However in this study, we found that in addition to the above three factors, vascular invasion and poor differentiation of tumors were also risk factors

Table 1 Correlation of E-cadherin expression to lymph node metastases in the 135 patients with early gastric cancer

Lymph node involvement	Cases	Loss of E-cad expression [cases (%)]	*P*
Features of involvement			
Negative	98	47(47.9)	
Single or scattered cells	26	19(73.1)	<0.001
Clusters of cells	11	11(100)	
Location of involvement			
Group 1	22	16(72.7)	<0.01
Group 2 or 3	15	14(93.3)	
Number of CK-positive nodes			
1	13	9(69.2)	
2-3	17	14(82.4)	<0.01
≥4	7	7(100)	

of lymph node metastasis for gastric cancer. A traditional point of view was that the probability of lymph node metastases in tumors less than 2.0 cm in diameter was low [2, 6]. Our study found that tumors with the diameter of greater than 2.0 cm more easily emerged lymph node metastases, and then the majority of cancer cells were in the form of clusters in the metastatic lymph nodes. However, we also found that tumors with the diameters between 1.0 cm and 2.0 cm in the 5 cases of mucosal cancers and 4 cases of submucosal cancers had lymph node micrometastases, although these micrometastatic cancer cells were in the form of a single cell or scattered cancer cells in the lymph nodes.

An interesting phenomenon was that 5 cases of mucosal cancer with micrometastases involved only group 1 lymph nodes; but in 4 cases of submucosal cancer with micrometastases, 3 cases involved group 2 and/or group 3 lymph nodes. This was similar to the findings proposed by Yasuda *et al.*[16] that tumors with diameter of 1.0-2.0 cm in had the possibility of lymph node metastasis. These results indicate the risk of lymph node metastasis when the primary sites of early gastric cancer were more than 1.0cm in diameter,

The idea that tumors with deep and lymphatic invasions are more easily to have lymph node metastases has been widely accepted [17,18].

Using CK immunohistochemical staining, we found that the rate of lymph node involvement in mucosal cancer was 19.0%, and that in submucosal cancer was 34.6%. In most of the mucosal cancer with lymph node micrometastases, cancer cells were in the form of a single cell or scattered cells in metastatic lymph nodes; while in most of the submucosal cancer with lymph node micrometastasis, cancer cells were in the form of clustered or scattered cells in metastatic lymph nodes. These suggest that the deeper the invasion, the more cancer cells metastasizing to the lymph nodes. Moreover, in the patients with lymphatic invasion, 66.7% were found the presence of micrometastases. Multivariate analysis showed that lymphatic invasion was an independent risk factor for lymph node micrometastases. The main reason of lymph node metastases in gastric cancer is that there is a large lymphatic network in the submucous layer of the gastric wall [17. 19]. When cancer cells invade to the submucosa, they can easily intrude into the rich lymphatic network, and then metastasize to the further distance. We suggest that lymphadenectomy should be recommended for some patients.

E-cad gene is generally considered as a metastastic suppressor gene [12,20], and it plays an important role in the maintenance of the normal epithelial tissue structures. The loss of its expression leads to the risk potential in tumor metastases [21,22]. We found that E-cad had normal expression in the normal gastric mucosa adjacent to the primary cancer sites. Similar to the results of Bolk *et al.* [23] that there was abnormal expression of E-cad in 57.0% of the primary sites of early gastric cancer. Poor differentiation, deep invasion, tumor more than 1.0 cm in diameter, lymphatic and vascular invasion are risk factors for the loss of E-cad expression, where were also closely correlated to lymph node metastases as detected by CK immunohistochemical staining. In the 45 patients with poorly differentiated or undifferentiated types of early gastric cancer, 40 patients (88.9%) had decreased expression of E-cad. Lymph node metastases of the 40 cases mostly belonged to group 2 and/or group 3 lymph nodes, but not group 1, with a large number of clustered cancer cells in metastatic lymph nodes. Of all patients found lymph node micrometastases by CK immunohistochemical staining, 81.1% existed abnormal expression of E-cad. The abnormal expression of E-cad led to a loss of inhibition of metastasis, so that the tumor cells were more easily to shed from the primary sites to lymph nodes [20,22]. The abnormal expression of E-cad may be one of the important risk factors of lymph node metastasis in early gastric cancer [24, 25].

In this study, of 43 early gastric cancer patients with CK-positive lymph nodes, 28 (65.1%) showed a single cell or scattered cancer cells in the metastatic lymph nodes. Although it is not clear whether the single cell or scattered cells in the lymph nodes would grow, or would be removed by immune defense system [10], 73.1% of these cases revealed abnormal expression of E-cad in the primary sites. Multivariate analysis showed that the abnormal expression of E-cad in the primary sites is an independent risk factor of micrometastases. The 5-year survival rate of patients with lymph node micrometastases was significantly shorter than that of those without micrometastases, implying that the influence of a single cell or scattered cancer cells micrometastasizing to lymph nodes may have the same effect as clustered cancer cell metastasis, which would affect the 5-year survival rate of the patients.

In recent years, endoscopic mucous resection or laparoscopic gastric resection has tended to be performed for the treatments of early gastric cancers. We suggest that endoscopic mucosal resection or laparoscopic wedge resection of gastric without lymphadenectomy should be applied only to the gastric mucosal cancer patients of less than 1.0 cm in diameter, because lymph node metastases in these patients are extremely rare; when the size of gastric mucosal cancer is from 1.0 to 2.0 cm in diameter, D1 lymphadenectomy should be performed, because lymph node metastases often involve in group 1 lymph nodes, rarely in group 2 or group 3 in these patients; for the gastric mucosal cancer patients with the tumor of more than 2.0 cm in diameter, D2 or selective D3 lymphadenectomy should be performed, because these patients have the potential dangers of micrometastases in group 2, even in group 3 lymph nodes. Preoperative E-cad immunohistochemical staining of the samples extracted from endoscopic biopsy would help to assess the lymph nodes involvement in patients with early gastric cancer.

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